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„The proboscis morphology of frog-biting midges (Corethrellidae)"

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Contents

Abstract

Not all blood-sucking dipterans are flying pests, at least for humans. For example, the females of the family Corethrellidae exclusively feed on frogs for their blood meals. While the morphology of the feeding apparatus is well studied in those species that have big impacts on human health, like the mosquitoes, the frog-biting midges, which belong to the same infraorder as mosquitoes, Culicomorpha, received less attention from the scientific community. Especially the morphology of the blood-sucking proboscis of Corethrellidae has not been studied in detail with up-to-date imaging techniques. For this purpose, semi-thin sections of three species from genus *Corethrella*, *C. ranapungens*, *C. amazonica* and *C. peruviana* have been compared. A 3D-model of the proboscis of *C. peruviana* was reconstructed using the computer software AMIRA. The morphological analysis of the three species has been complemented using Scanning Electron Microscopy and Light Microscopy. The results were subsequently compared to species of the other blood-sucking dipteran families Culicidae, Simuliidae, Ceratopogonidae and Psychodidae to find similarities and differences in the morphology and function of the proboscis. The three *Corethrella* species show a very similar proboscis morphology, when compared to piercing sucking representatives of other dipteran families. They share the same components and principal morphology of the mouthparts that make up the proboscises. However, the Culicidae have much longer proboscises and the salivary duct and food canal show major differences to the other blood sucking Diptera. Sensilla on the labella and maxillary palps of the three species of *Corethrella* are described and their presumable function is compared to the results to other studies focusing on proboscis sensilla in other nematoceran flies. The present work also discusses the miniaturization of mouthparts and possible evolutionary constraints that may limit the size, like the structural solidity of piercing structures and dimensions of anuran blood cells.

Key words: Insects, Diptera, piercing mouthparts, blood-feeders, miniaturization, sensilla

Introduction

Feeding behavior of Corethrellidae

Part of the success and diversity of the insects stems from a highly variable morphology and a big variety of niches they occupy. This is especially true for the type of food source they are specialized on. This specialization is made possible by many different modifications of the insects´ mouthparts. The plesiomorphic orthopteroid or biting-chewing functional type of the mouthparts has been adapted to other forms of food uptake in the different insect taxa. Even though their form and function may vary a lot, the set of mouthparts always consists of the same principal components (Snodgrass, 1935).

The females of the family Corethrellidae Wood & Borkent, 1989, have adapted to obtain blood meals exclusively from anuran hosts. In addition, both the females and the males need nectar from flowering plants to sustain themselves, but most female frog-biting midges need blood to produce their eggs (McKeever, 1977; McKeever & French, 1991). Like other blood sucking flies the members of Corethrellidae use a variety of signals to find their hosts such as olfactory, visual, air humidity, temperature, and tactile indicators. In addition to those they use auditory cues from their hosts to locate them at further distances. This adaptation has likewise been observed in other families of Diptera, such as Tachinidae and Sarcophagidae (Bartlett-Healy et. al., 2008). In the case of Corethrellidae, they exploit the mating calls of male frogs to find their next blood meal. They probably perceive the calls with their Johnston's organs on their antennae (Borkent & Belton, 2006). After hearing the frogs´ mating calls from a distance, some land directly on their hosts while other species usually land nearby on the ground or vegetation to approach their hosts by walking or short flights (Bernal et al., 2006; Borkent, 2008). The preferred feeding site on the frogs seems to depend on the amount of vascularization of the tissue. The areas around the nostrils, the thoracic dorsum (Bernal et al. 2006; de Silva et al., 2014) and the legs (McKeever, 1977) are often preferred feeding sites. The selection of the right feeding site is an important decision and is always a trade-off between the desire to maximize blood intake and reduce the costs, such as risk of injury (de Silva et al., 2014). If undisturbed Corethrellidae tend to feed until they are saturated, then being unable to fly they just walk off their anuran hosts to expel enough fluid to be able to resume flight (Borkent, 2008). Their preferred time for looking for blood meals seems to be during the night, which obviously must match the time when male frogs are actively calling for mates (Toma et al., 2005). Female Corethrellidae showed high preferences in playback experiments when it came to certain acoustic properties of the mating calls, such as the frequency and rate of the calls (Meuche et al., 2016; Ambrozio-Assis et. al., 2019; Virgo et al., 2019; Legett et al., 2021). A certain degree of host specificity is also likely, although not sufficiently investigated yet (Borkent, 2008).

Phylogeny & distribution

The family Corethrellidae belongs to the dipteran infraorder Culicomorpha, forming the sister group to the phantom midges (Chaoboridae), the meniscus midges (Dixidae) and the mosquitoes (Culicidae) (Saether, 2000). The Corethrellidae includes only the single genus *Corethrella* Coquillett, 1902, containing 115 extant species, even though it is very likely that there are many more to be found and described (Almeida, 2021).

The distribution of *Corethrella* species is centered around tropical and subtropical regions, with highest densities and diversities in lowland areas and decreasing rapidly at higher altitudes. Only a few more distant islands are inhabited by Corethrellidae, which suggests relatively limited dispersal capabilities. The abundance of anuran hosts is an important limiting factor for their worldwide distribution (Borkent, 2008).

Development

Some days after the blood meal a female *Corethrella* lays eggs on a water surface. A few days later the larvae hatch, all together the adult matures from the egg in about one month. The larvae develop in small aquatic habitats like small pools or swamps, but most often in so-called phytotelmata (Borkent, 2008). These are small water-filled pools in plants, mostly formed by bromeliads, pitcher plants, tree holes and bamboo internodes (Kitching, 2000). The larvae are ambush predators of other insect larvae, crustaceans, or nematodes, catching the prey with their antennae and strong mandibles. As such they seem to be an important factor of community structure in their habitats (Borkent, 2008).

Feeding apparatus

Like other blood sucking flies, the organs of feeding consist of the proboscis and two sucking pumps, the cibarial pump beneath the clypeus and the pharyngeal pump in the posterior of the head. The proboscis is formed by all typical components found in most insects' mouthparts: the labrum, a pair of mandibles, a hypopharynx, a pair of maxillae and a labium. These structures are modified from the biting-chewing mouthparts (Snodgrass, 1935), that represent the plesiomorphic functional type in insects (Krenn 2019) and enable the frog-biting midges to suck blood and/or nectar. The labium encloses the other structures of the proboscis that pierce the skin during blood-sucking, forming a sheath for the so-called stylets. The food canal, through which the food is sucked up, is formed by the labrum and the mandibles while the salivary canal is contained within the hypopharynx and is used to inject saliva to stop the hosts blood from coagulating (Snodgrass, 1959; McKeever & Pound, 1979). Since the males do not feed on blood, these structures are often highly reduced or absent all together (McKeever, 1986).

Fluid mechanics

To be able to suck up liquids, i.e. nectar or blood, the animal must exert negative pressure inside the food canal. This is done by contraction of the dilator muscles of the cibarial and pharyngeal pump, thus expanding the pumping chamber. The Hagen–Poiseuille equation states that the flow rate of a liquid through a tube is inversely proportional to the viscosity of the fluid and the length of the tube, but directly proportional to its radius (Lee et. al., 2009). This means that these factors together with the pumping capacity of the pumps limit the amount of fluid taken up by the animal per time unit. With the viscosities of the fluids being constant, the dimensions of the food canal and the pumps are the levers where evolution can apply change to ensure optimal fluid uptake. Optimizing the fluid uptake is, like the afore mentioned feeding site selection, important to maximize the benefit/cost relation. This is especially true for blood-feeding insects since the rate of blood intake is directly related to the egg production of female Culicidae (Kim et. al, 2011).

Risk and trade-off for anuran hosts

The risk of predation or parasitism for anurans due to interception of their mating calls is compensated by increased reproductive success. A calling male frog can attract several hundred female Corethrellidae (Bernal et al., 2006), which can result in blood loss that can have a negative impact on the anurans. In addition to that, the female Corethrellidae can be vectors of disease and can infect the frogs with parasites like trypanosomes. Such an infection can be pathogenic, further decreasing the fitness of the anuran hosts and making the mating calls even more costly for males (Diamond, 1958; Bardsley & Harmsen, 1973). It has also been suggested, that the chytrid fungus (*Batrachochytrium dendrobatidis*) might also be transmitted by Corethrellidae (Toledo et al., 2021). This fungus is a skin pathogen that can trigger chytridiomycosis, which is considered as one of the biggest threats to amphibians (Olson et al., 2021). If this proves to be true it could spell disastrous news for the already endangered anuran fauna in regions with populations of Corethrellidae.

Aim of this study

The objective of this work is to examine and describe the proboscis morphology and anatomy of the feeding apparatus of three species of Corethrellidae and to complement data from existing literature with up-to-date micromorphological techniques. These findings have been compared to other blood-sucking insects, including species of various families of the Culicomorpha. Functional adaptations to blood sucking from anuran hosts will also be explored. Different imaging methods were used to obtain the data needed for the investigation of proboscis and feeding apparatus morphology. To get a detailed representation of the interior anatomy of the proboscis and feeding apparatus, semi-thin sections of the animals were made, which were then analyzed using a computer software to create a 3D-reconstruction of the proboscis.

Material & methods

Studied species

The animals investigated were kindly provided by Jonas Virgo and his colleagues from the Ruhr-University Bochum after being caught using acoustic traps in Costa Rica at the tropical station La Gamba (Golfito/Puntarenas, 83°12'7"W, 8°42'2"N; 77 m asl) and fixated in alcohol. Three *Corethrella* species, *C. ranapungens* Borkent, 2008, *C. amazonica* Lane, 1939*, and C. peruviana* Lane, 1939, were studied.

Semi-thin sections and 3D-reconstruction

For the semi-thin sections, the insects (*C. ranapungens, C. amazonica, C. peruviana*) were first dehydrated in ascending alcohol concentrations (three times 15 minutes in 70 %, 15 minutes in 80 %, 15 minutes in 90 %, 15 minutes in 96 %, three times 15 minutes in 100 %) and 100 % acetone at room temperature. The specimen were then embedded in Agar Low Viscosity Resin (Agar Scientific Ltd, Essex, UK) after gradually increasing the concentration of the resin (50 % : 50 % acetone to resin overnight, 30 % : 70 % acetone to resin for 6 hours, 100 % resin overnight). After 2.5 hours in a vacuum chamber at 150 mbar at 40 °C and hardening for 24 hours at 70 °C the resulting blocks were then trimmed to an appropriate size and cut into 1 µm sections with a microtome (Leica EM UC6, Leica GmbH, Wetzler, Germany). The sections were subsequently mounted onto glass slides, stained with Toluidine blue (0.1 %), embedded in Agar Low Viscosity Resin and covered with cover glasses. Photos of the sections were taken using a microscope (Nikon Eclipse E800, Nikon, Tokyo, Japan) equipped with a digital camera (Nikon DS-Ri2, Nikon, Tokyo, Japan) at 400x magnification in oil immersion which were then partly converted into greyscale using Adobe Photoshop (CC 2018, California, USA). The alignment of the slices, segmentations and surface renderings for the 3D-reconstruction were done using the computer software Amira (6.4.0, Thermo Fisher Scientific, Massachusetts, USA).

Photo stacks

Whole mount slides were prepared by transferring the animals in glycerol and putting them onto glass slides with the cover glass being fixated using nail polish. Picture stacks of the specimen were taken using a microscope (Nikon Eclipse Ni-U, Nikon, Tokyo, Japan) with a digital camera (Nikon DS-Ri2, Nikon, Tokyo, Japan) by stacking together several pictures of different focal planes.

Scanning electron microscopy

The preparation of the insects for the scanning electron microscope started with the dehydration in an ascending alcohol series (30 minutes in 30 %, 30 minutes in 96 %, three times 10 minutes in 100 % at room temperature). To increase the durability of the animals against the electron beam they were put in hexamethyldisilazane (HMDS) for 15 minutes at room temperature. The animals were subsequently mounted onto the mounting tables using carbon platelets, conductive silver was used

where necessary to ensure even electron flow and thus reduce random noise. Finally, the specimen were coated with a thin gold layer using a Sputter-coater (JEOL JFC-2300HR, JEOL Ltd., Tokio, Japan). Photos of the specimens were taken using the scanning electron microscopes Philips XL 30 ESEM (Koninklijke Philips N.V., Amsterdam, Netherlands) at 15 kV voltage and JEOL JSM-IT300 (JEOL Ltd., Tokio, Japan) at 20 kV voltage.

Photos were edited and labelled using the Fiji plug-in ScientiFig. Measurements of sensilla lengths have been made using scanning electron microscopic photos in 5 specimens.

Results

Mouthpart morphology of the examined *Corethrella* species

The proboscises of the investigated females of *Corethrella, C. peruviana, C. amazonica* and *C. ranapungens*, show a similar morphology. The length of the proboscis ranges from 115 to 163 µm in the studied specimens (Tab. 1). The proboscis forms a piercing organ in females that is made up of all the typical components found in most nematoceran Diptera. Below the clypeus, which bears countless short microtrichia and a low number of long setae, is the labrum. The labrum, which does not bear any setae, becomes smaller and more slender toward the tip of the proboscis and forms the dorsal border of the food canal (Figs. 1-3). At the apex of the proboscis the labrum splits into two parts, called labral pegs (Fig. 1E). The mandibles reach to the very tip of the proboscis and lie very close to each other between the labrum and the hypopharynx (Fig. 1C). One mandible forms the ventral border of the food canal while the other functions as the dorsal border of the salivary groove (Fig. 3). The serrations on the mandibles, used to pierce the skin of the host, are especially evident in Figure 1E. The labium is composed by the prementum in the proximal regions of the proboscis and splits up into paired labella and an unpaired ligula in the distal regions. Here, it forms a sheath surrounding the stylets, which function as the piercing organs of these insects (Fig. 2). Numerous long setae can be seen on the labella, which bear grooves and smooth ridges along their longitudinal axis. The bristles have pointed tips, extend from a socket and are about 25 µm to 36 µm long. There are also shorter setae with a very similar appearance to those, measuring about 11 µm to 14 µm. Short, curved setae without such grooves or sockets are present on the labella in significantly higher abundance, measuring about 2 µm to 5 µm (Fig. 4A). The hypopharynx in the proximal regions of the proboscis lies dorsally of the prementum and then reaches into the prementum to envelop the salivary canal (Fig. 3A, B). More distally, the salivary canal transitions into the salivary groove, which is formed by the hypopharynx (Fig. 3C, D). Here, the hypopharynx becomes very slender and pointed; it has the shape of a thin stylet. In the proximal regions of the proboscis the salivary canal becomes thick-walled and has a pumping function, used to pump saliva into the host (Fig. 3B). The laciniae, as parts of the maxillae, form lateral

borders of the food canal in the proximal regions but do not reach further than two thirds towards the tip of the proboscis (Fig. 3A, B).

The maxillary palps are not part of the piercing apparatus itself. They consist of four segments, the first one seems to be made up of two mostly fused subsegments (Fig. 2). The surface of each segment bears long socketed setae with grooves and serrated ridges along its longitudinal axis which occur in two different length types. The longer setae measure between 93 µm and 143 µm, whereas the shorter ones vary in length from 22 µm to 48 µm. The surface of the maxillary palps is also covered with a high number of very short and smooth setae, all of which measure about 2 µm to 5 µm (Fig. 4B). The second segment is elongated and has blunt tipped sensillae towards its distal end. These sensillae vary in their form and size in the three investigated species of *Corethrella*. While they are club-shaped in *C. peruviana* (15 µm length; Fig. 4C) and *C. ranapungens* (12 µm length; Fig. 4D), these sensillae have a spoon shape in *C. amazonica* (8 µm length) and are more numerous and more tightly packed together (Fig. 4E, F).

Proboscis morphology in 3D reconstruction

In the 3D-model of the proboscis in *C. peruviana* (Fig. 5), the labella of the labium do not envelop the other stylets like they usually do in the live animal. This is regarded to be an artifact. The position of the stylets and the length proportions of all the components however is true to the presumably normal state. It is evident that all the stylets, except for the laciniae, reach to the very tip of the proboscis. The laciniae reach only up to two-thirds towards the tip of the proboscis and form the lateral borders of the food canal in the middle to proximal regions. They appear as slender stylets that converge quickly towards their distal ends, making them less pointy than the mandibles. The two mandibles lie between the labrum and the hypopharynx and lie very close to one another. The labrum transitions into the bulging clypeus near the proximal third of the proboscis. The transition of the salivary canal into the salivary groove can be seen near the midsection of the proboscis. The salivary groove appears as a ridge on the ventral side of the hypopharynx. The two mandibles converge medially and lie closely together between the labrum and the hypopharynx. These three stylets show a kink towards the tip of the proboscis.

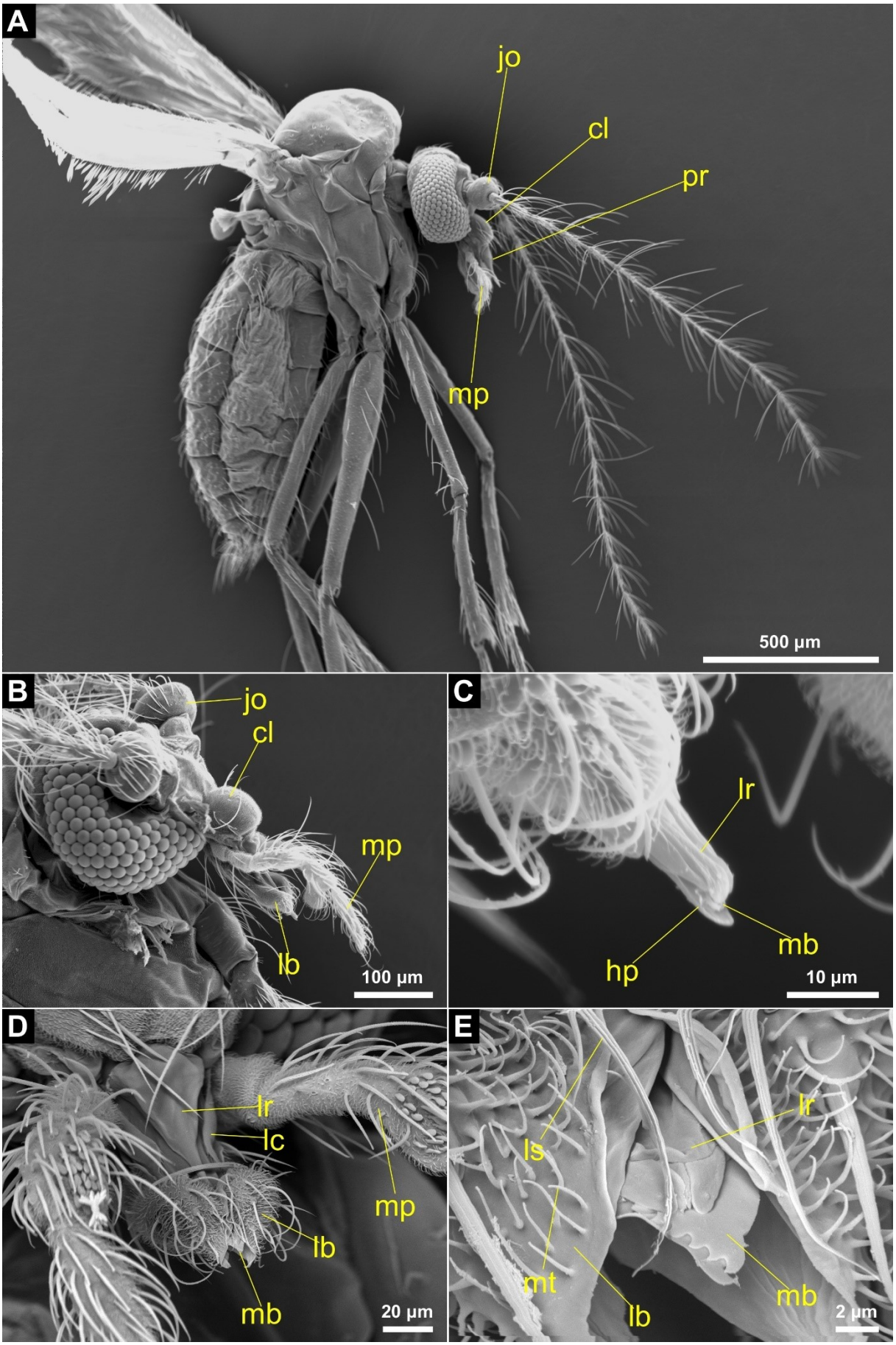
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Figure 1: Scanning electron microscopic images of *Corethrella* sp. (Corethrellidae, Diptera). A: Lateral overview of *Corethrella peruviana.* **B:** Head and proboscis of *Corethrella ranapungens* showing the Johnston´s organs at the base of the antennae, clypeus, and maxillary palps. **C:** Proboscis tip of *Corethrella ranapungens* highlighting the mandibles positioned between labrum and hypopharynx. **D:** Maxillary palps and proboscis of *Corethrella amazonica* showing the labella forming a sheath for the stylets. **E:** Proboscis tip of *Corethrella amazonica* highlighting the serrations on the mandibles, split labrum tip as well as microtrichia and grooved setae on the labella. cl: clypeus; hp: hypopharynx; jo: Johnston´s organ; lb: labellum; lc: lacinia; lr: labrum; ls: labellar seta; mb: mandible; mp: maxillary palp; mt: microtrichium; pr: proboscis.

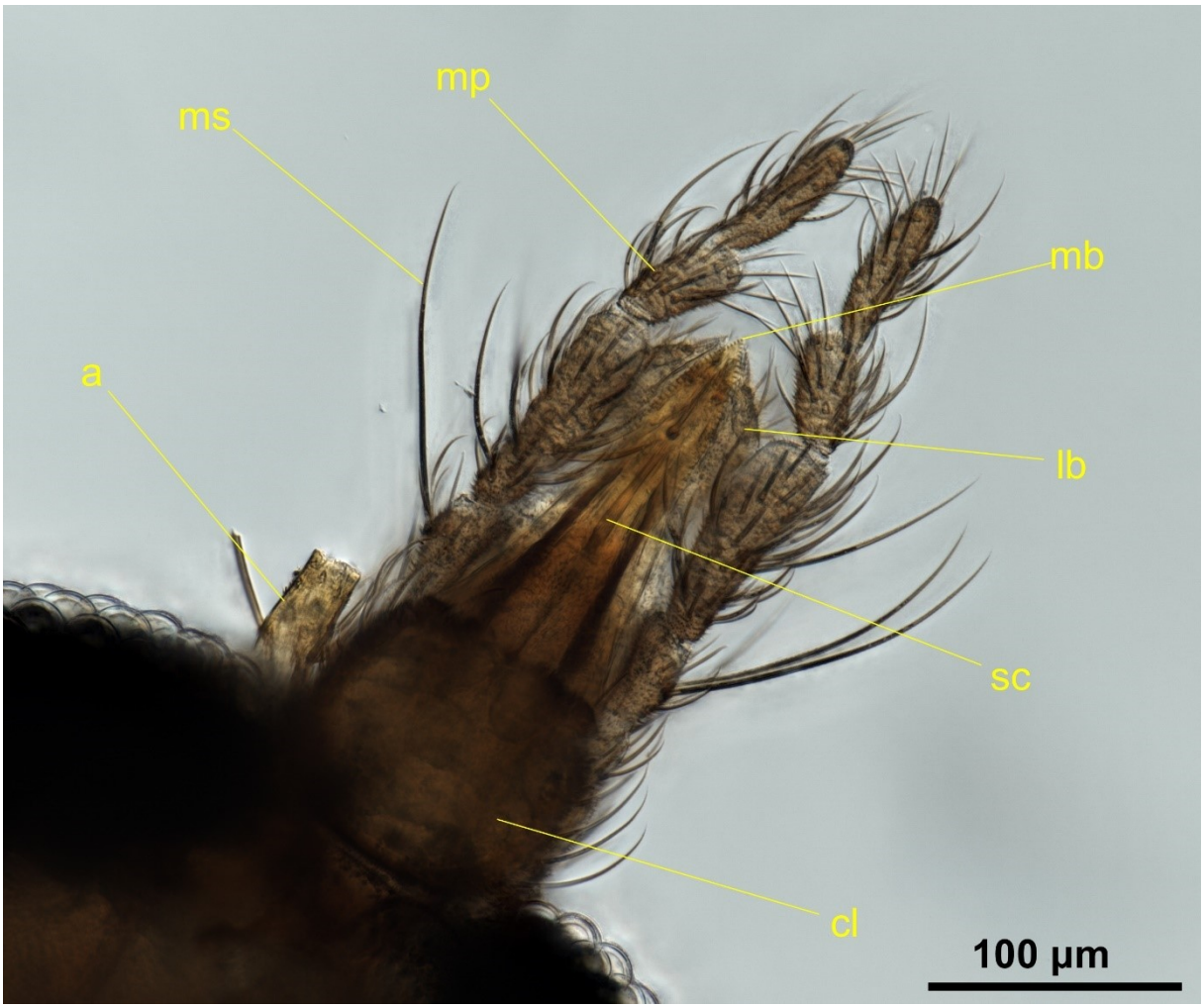

Figure 2: Light microscopic image stack of the proboscis and maxillary palps of *Corethrella amazonica.* Maxillary palps bear setae and consist of four segments with the second being elongated. Labella envelop the stylets of the proboscis. Serrations on the mandibles are visible. a: stump of cut-off antenna; cl: clypeus; mb: mandible; mp: maxillary palp; ms: maxillary palp seta; sc: salivary canal.

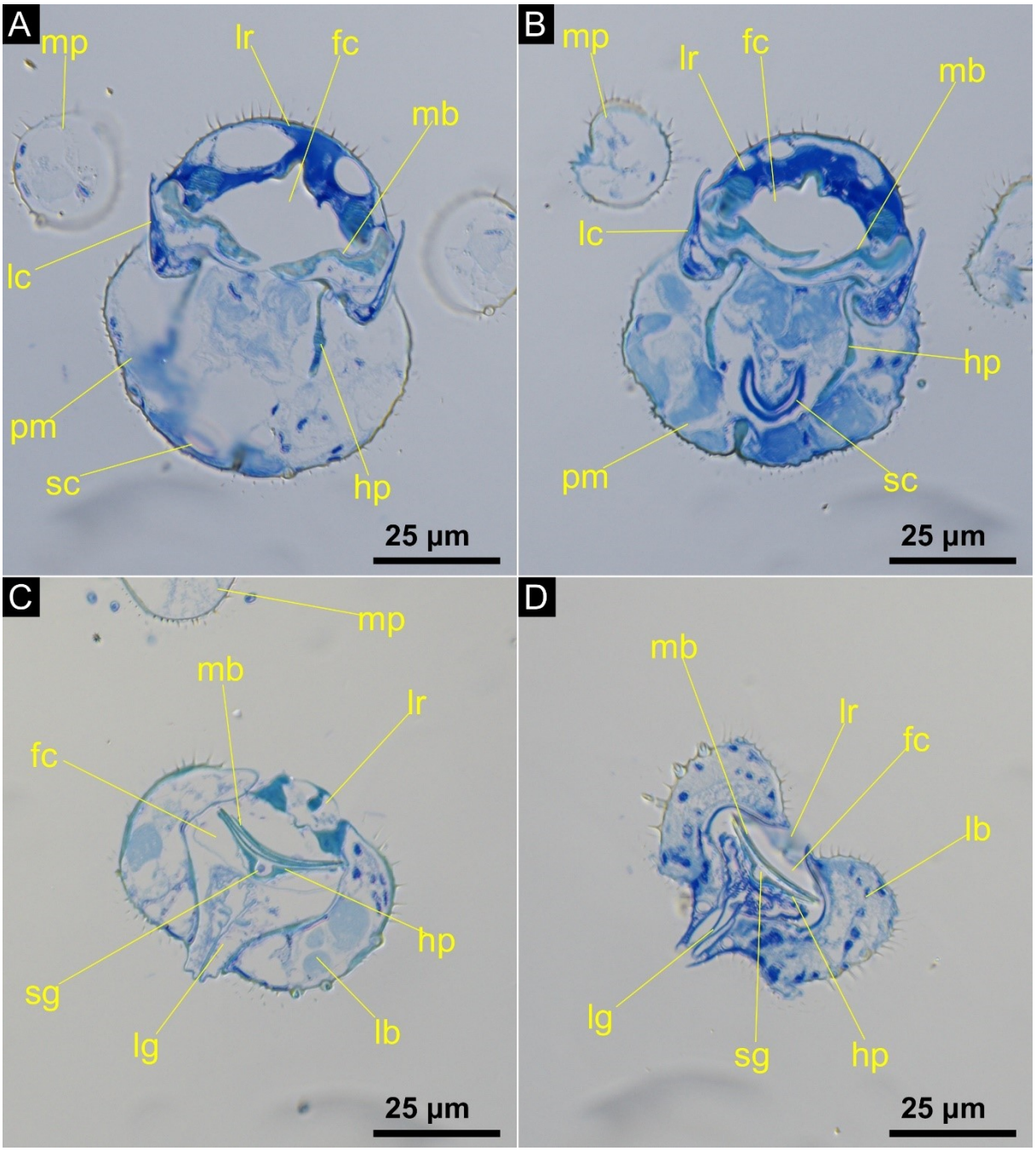

Figure 3: Light microscopic micrographs of semithin cross-sections of the proboscis of *Corethrella peruviana.* In the proximal region the thin-walled salivary canal (**A**) transitions into a thicker-walled salivary canal (**B**) and then into the salivary groove in the distal region of the proboscis (**C, D**). One mandible forms the roof of the salivary groove, which is shaped by the hypopharynx. Mandibles and hypopharynx bound the food canal alongside the labrum. The laciniae form the lateral borders of the food canal in the proximal region. As part of the labium, the praementum splits into two labella and the ligula, enveloping the stylets of the proboscis. fc: food canal; hp: hypopharynx; lb: labellum; lc: lacinia; lg: ligula; lr: labrum; mb: mandible; mp: maxillary palp; pm: praementum; sc: salivary canal; sg: salivary groove.

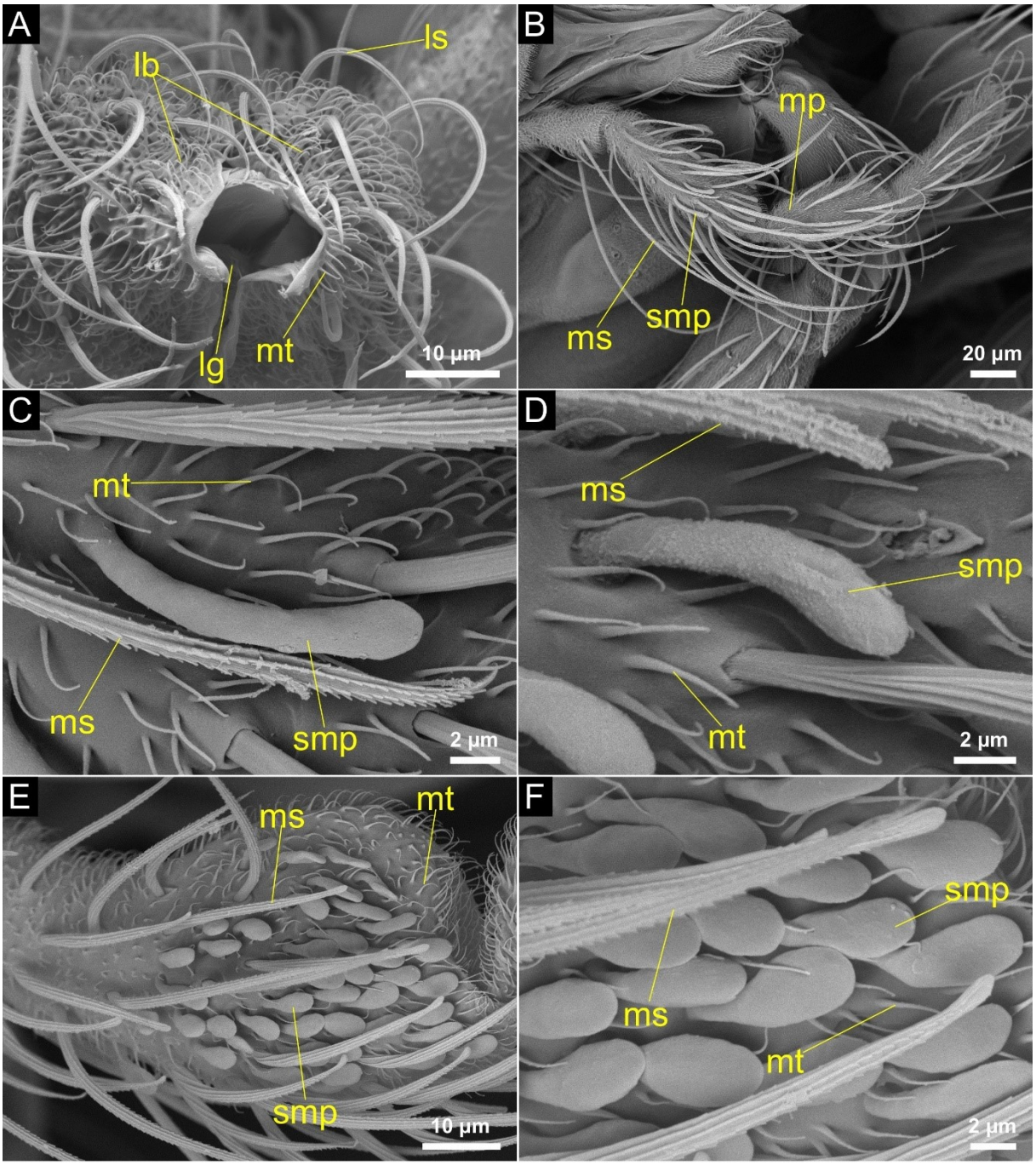

Figure 4: Scanning electron microscopic images of microtrichia, setae and sensilla of *Corethrella* sp. A: Proboscis tip of *Corethrella amazonica* with microtrichia and socketed setae covering the labella. Ligula visible between the labella. **B:** Overview of the four-segmented maxillary palp of *Corethrella peruviana* with maxillary palp setae and club-shaped maxillary palp sensilla. **C:** Detail of microtrichia, grooved, socketed and serrated setae and club-shaped sensilla on the maxillary palp of *Corethrella peruviana.* **D:** Detail of microtrichia, grooved, socketed and serrated setae and club-shaped sensilla on the maxillary palp of *Corethrella ranapungens*. **E, F:** Detail of microtrichia, grooved, socketed and serrated setae and spoon-shaped sensilla on the maxillary palp of *Corethrella amazonica*. lb: labellum; lg: ligula; ls: labellar seta; mp: maxillary palp; ms: maxillary palp seta; mt: microtrichium; smp: maxillary palp sensillum.

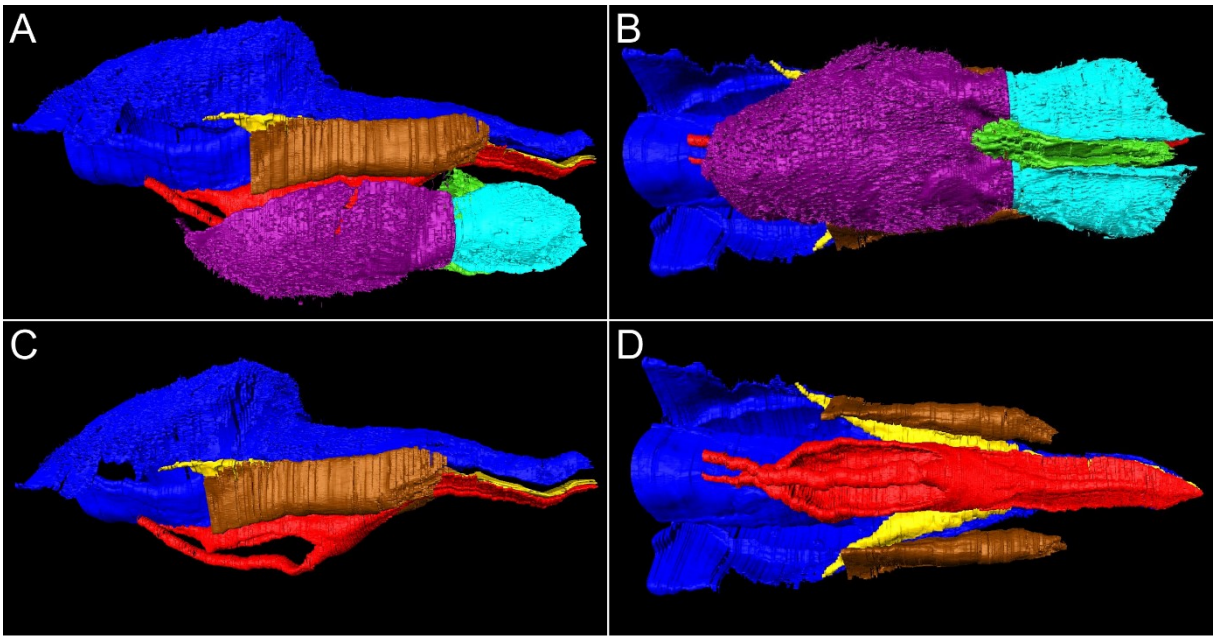

Figure 5: 3D-model of the proboscis of *Corethrella peruviana*. Tip of the proboscis points to the right side. The whole 3D-model represents 263 µm. All the stylets, except for the laciniae, reach the very tip of the proboscis. Bulging clypeus transitions into flat labrum around the midsection of the proboscis. **A:** Lateral view; note that the labella do not form a sheath for the stylets in this specimen. **B:** Ventral view; praementum splits into labella and ligula towards the proboscis tip. **C:** Lateral view with removed praementum, labella and ligula; salivary canal fuses with hypopharynx and transitions into salivary groove near the midsection of the proboscis. **D:** Ventral view with removed praementum, labella and ligula; salivary groove is visible in the distal third as ridge on the ventral side of the hypopharynx. Dark blue: clypeus and labrum; brown: laciniae; green: ligula; red: hypopharynx and salivary canal; turquoise: labella; violet: prementum; yellow: mandibles.

Discussion

Comparison of the proboscis of Corethrellidae to other blood-sucking Diptera

Insects show a high degree of adaptation to the kind of food sources they use to nourish themselves. The biting-chewing function of the mouthparts has been adapted to other ways of feeding, and for this the same set of mouthparts has seen morphological modifications in the different insect groups (Snodgrass, 1935). In the case of blood-feeding insects, functional adaptation led to piercing-sucking mouthparts through convergent evolution, which form a proboscis that enables the insects to pierce through the skin and to suck up liquids, like blood among other body fluids. Even though the proboscises of different insect taxa are often made up of varying structures and can show different functional mechanisms, they still share similarities like puncturing, penetrating and anchoring structures, a sheath-like covering and most of the time a food canal which is separated from a salivary canal (Krenn & Aspöck, 2012).

The main goal of this work was to analyze the structures that make up the piercing blood-sucking proboscis of the Corethrellidae using modern imaging techniques, complementing the findings from existing literature. All investigated species display a very similar proboscis morphology that also matches what was found in the previous studies (McKeever & Pound, 1979; McKeever, 1986). In the 3D-reconstruction of the proboscis of *C. peruviana,* a kink in the labrum, hypopharynx and mandibles can be observed. If this turns out to be the normal physiological state, it could increase the rigidity and stiffness of these stylets and assist the animal in the stinging process. One adaptation of the proboscis of Corethrellidae to their anuran hosts are the serrated mandibles in conjunction with the unarmed laciniae. Borkent (2008) points out, that this combination of traits occurs also in species of Psychodidae and Ceratopogonidae, all of which obtain their blood meals from anuran hosts. He states that this may be the case because of the loose skin of the frogs, which is why the anchoring function of the laciniae is not needed as it is in other blood sucking Diptera that have different vertebrate hosts.

Differences and similarities to proboscises of other blood sucking dipterans have been investigated. The compared species of the three taxa Phlebotominae (Silva & Grunewald, 2000; Krenn & Aspöck 2012), Simuliidae (Wenk, 1962; Sutcliffe & McIver, 1984; Krenn & Aspöck, 2012) and Ceratopogonidae (McKeever et al., 1988; Krenn & Aspöck, 2012) show a similar proboscis morphology compared to the Corethrellidae although they are not closely related with the frog-biting midges. These taxa were chosen for this comparison because, like the Corethrellidae, their representatives are rather small. The food canal is bordered dorsally by the labrum and ventrally by the mandibles, with the hypopharynx lying even further on the ventral side. It is likely however, that the hypopharynx also plays an important role in forming the food canal in conjunction with the mandibles. This is necessary because the movement of the mandibles during the puncturing process of the host's skin jeopardizes the sealing of the food canal. In Simuliidae (Sutcliffe & MrIver, 1984) and Ceratopogonidae (McKeever et al. 1995), the mandibles perform a snipping motion to open the skin of the host. Even though the exact mechanism of the skin-piercing process in Corethrellidae is unknown, the absence of an interlocking mechanism as well as the lack of serrations on the laciniae seem to indicate that the mandibles do not move in such a way (McKeever, 1986; de Silva et al., 2014). But they certainly do move during the piercing process, which probably makes an additional sealing function of the hypopharynx necessary. The labium forms a sheath for the stylets of the proboscis.

Some interesting differences of the proboscis morphology compared to the three taxa described above are found in the family Culicidae. Firstly, the proboscises are a much longer than those of *Corethrella*. The food canal is mainly formed by the labrum and is bordered ventrally by the hypopharynx, but the mandibles and laciniae lie outside of the food canal and not in between the labrum and the hypopharynx like they do in the other three taxa (Robinson, 1939; Snodgrass, 1959; Hudson, 1970;

Krenn & Aspöck, 2012). In Corethrellidae (McKeever & Pound, 1979; McKeever, 1986), Ceratopogonidae (McKeever et al., 1988), Phlebotominae (Silva & Grunewald, 2000; Krenn & Aspöck, 2012) and Simuliidae (Sutcliffe & McIver, 1984; Krenn & Aspöck, 2012) the mandibles (and the hypopharynx) together with the labrum form the food canal and are surrounded by the labrum and the hypopharynx. In these four taxa the salivary canal opens up and transitions into the salivary groove towards the tip of the proboscis. In the distal region of the proboscis of these taxa one mandible forms a border of this salivary groove and prevents saliva from leaking into the food canal (McKeever & Pound, 1979). This also enables the animal to pump saliva into the host during the blood-sucking process while simultaneously sucking up the liquid through the food canal (Sutcliffe & McIver, 1984; McKeever, 1986; McKeever et al., 1988). The salivary canal of Culicidae is closed up to the tip where it releases salivary fluid during the piercing process (Robinson, 1939; Snodgrass, 1959; Hudson, 1970; Wahid et al., 2007; Krenn & Aspöck, 2012).

Sensilla of labella and maxillary palps

Three different types of sensilla on the labella and the maxillary palps have been observed in the investigated species of *Corethrella*. The long setae present on the maxillary palps are similar in structure to the sensilla chaetica found on the antennae of two species of *Anopheles* (Culicidae) (Pitts & Zwiebel, 2006). Their long, grooved appearance as well as their placement in a socket suggest a function as a mechanoreceptor (McIver, 1975). No pores or openings, which would suggest a chemosensory function, could be observed using the scanning electron microscope. Two types of long setae have been found on the labella of the investigated species, differing in size. Both types resemble the long setae found on the maxillary palps, showing similar grooved surfaces and a socket. Lee & Craig (2009) also found two length types of long setae on the labella of *Aedes aegypti* (Culicidae). They stated that the longer ones, which they called long labellar hairs, are mechanosensory and are probably responsible for checking the position of the labella on the hosts skin during the feeding process. They concluded that the shorter setae, which they called medium-sized hairs, are chemosensory and are used to probe the skin of the host to check for suitability. Spiegel and colleagues (2005) found three different length types of this kind of sensillum on the labellar lobes and maxillary palps of *Lutzomyia longipalpis* (Psychodidae) and described them as trichoid sensilla. Even though the authors of this study were also not able to detect pores or openings on the surface of these sensilla using scanning electron microscopy, they made an indirect proof of their existence by using a silver-staining method and light microscopy. This would indicate a chemosensory function of these sensilla.

The short, very abundant setae on the labella and the maxillary palps closely resemble the non-sensory microtrichia described by authors for species of Psychodidae (Spiegel et al., 2005) and Culicidae (Lee & Craig, 2009).

The club shaped sensillae on the second segment of the maxillary palps observed in the investigated species of *Corethrella* are similar to those found in species of Simuliidae (Nicholson, 1945) and Ceratopogonidae (McKeever et al., 1988; McKeever et al., 1995) as well as other species of Corethrellidae (McKeever & Pound, 1979). Spiegel et al. (2005) described similar sensillae on the maxillary palps of *Lutzomyia longipalpis* (Psychodidae) as capitate peg sensilla. A chemosensory function of these sensilla, especially a sensitivity for carbon dioxide, has been verified by other researchers in Culicidae (Grant & O´Connell, 1996; Steinbrecht, 1996). These sensilla would therefore assist the animal in locating its host by detecting the carbon dioxide exuded by it.

Comparison of mandible and hypopharynx width between families of blood sucking insects

When comparing the absolute dimensions of the mandibles and hypopharynx in relation to the width of the proboscis, measured by the width of the labium, of different blood sucking insects an interesting trend can be observed (Tab. 1). Even though the proboscis of *Anopheles stephensi* (Culicidae) (Krenn & Aspöck, 2012) is a lot wider than those of the other compared species, *Culicoides hollensis* (Ceratopogonidae) (McKeever et al., 1988), *Lutzomyia migonei* (Psychodidae) (Silva & Grunewald, 2000), *Corethrella peruviana*, *C. ranapungens* and *C. amazonica*, the widths of the mandibles and hypopharynx are quite similar. The mandibles of *Anopheles stephensi* are even narrower than those of the other animals. *Simulium* sp. (Simuliidae) is an exception to the trend described before. The proboscis is a lot wider than those of the other previously described species, as are the mandibles and the hypopharynx (Krenn & Aspöck, 2012).

A possible explanation for this trend is a size constraint for the stylets of the proboscis of blood sucking insects. There might be a lower size limit of these structures for them to still be able to perform their functions during the stinging process. The members of the families Corethrellidae and Ceratopogonidae are very small insects. During their evolution they probably underwent adaptations regarding the miniaturization of their body parts, including the lengths of stylets composing the short proboscis.

Table 1: Dimensions of the mandibles, hypopharynx, and proboscis of different species of Culicomorpha. Proboscis length was measured from distal end of the clypeus to the tip of the proboscis. Measurements are in micrometers.

	mandible width	hypopharynx width	labium width	proboscis length
Anopheles stephensi [1]	21,9	32,3	78,7	
Culicoides hollensis [2]	27	30	55	
Corethrella peruviana	23,9	26,4	66,5	163
Corethrella ranapungens	27,4	29,2	64,3	115
Corethrella amazonica	31,5	35,5	62,9	141
Phlebotomus duboscqi [3]	18,0	-	70,7	
Lutzomyia migonei [4]	17,1	28,3	-	
***Simulium* sp.** [5]	86,7	101,4	171,7	

References: [1]Krenn & Aspöck, 2012; [2]McKeever et al., 1988; [3]Krenn & Aspöck, 2012; [4]Silva & Grunewald, 2000; [5]Krenn & Aspöck, 2012.

Anuran red blood cell size – a constraining factor for miniaturization?

Anuran erythrocyte blood cells range in their longest diameter from 10.6 µm to 28.3 µm among different species worldwide (http://www.genomesize.com/cellsize/amphibians.htm; 03. October 2022, 11:45). Since in Corethrellidae the mandibles and the hypopharynx make up the food channel alongside with the labrum, the size of erythrocyte blood cells of the anuran host probably limits the miniaturization of the food canal of these mouthparts in Corethrellidae. The food canal must be wide enough to enable the blood cells to pass through during the feeding process.

Similarly, the erythrocyte blood cell size of mammals and birds may have a size constraining effect on the mouthparts of Simuliidae, Ceratopogonidae and Culicidae, which obtain their blood meals from mammals and birds among others. In birds, the longest diameter of erythrocyte blood cells ranges from 9.5 µm to 16.3 µm (http://www.genomesize.com/cellsize/birds.htm; 03. October 2022, 12:02). Mammals show longest diameters of erythrocyte blood cells between 2.1 µm and 10.8 µm (http://www.genomesize.com/cellsize/mammals.htm; 03. October 2022, 12:08).

This hypothesis has to be tested by comparing the diameters of the food canal of species of blood sucking dipterans to the diameters of erythrocyte blood cells of their confirmed host species.

Impact of labium length on feeding site selection

De Silva et al. (2014) described a relation between the vascularisation density and depth of blood vessels in the skin of the anuran hosts to the preferred feeding site of female Corethrellidae. For Túngara frogs (*Engystomops pustulosus*), where blood vessels are fewer and at a greater distance from

the epidermis on the thoracic dorsum, the preferred feeding site for the midges is around the nostrils, where the blood vessel density is higher, and the skin is thinner. In treefrogs (*Dendropsophus ebraccatus* and *Dendropsophus microcephalus)* this difference of vascularisation is a lot smaller, and the frog-biting midges fed not only from the area around the nostrils but also the thoracic dorsum and the legs. By measuring the length of the labium of species of Corethrellidae, the authors were also able to determine the potential depth in which they could reach the blood vessels. This also explains their preferred feeding sites on the different anuran hosts with varying vascular properties.

In the present study, the length of the proboscis of the three investigated species of *Corethrella* was determined by measuring the distance from the distal end of the clypeus to the tip of the labrum (Tab. 1). Since the clypeus is not inserted into the hosts skin during the feeding process this also marks the maximal insertion depth of the proboscis and thus the maximal depth in which blood vessels can be reached. If the specific anuran hosts of the studied *Corethrella* species were known and the sample size of measurements was bigger, the experimental framework of the study of de Silva et al. (2014) could be applied on the results of this work and the preferred feeding sites on the blood hosts could be inferred.

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Zusammenfassung

Nicht alle blutsaugenden Zweiflügler sind Plagegeister, zumindest für uns Menschen. Die Vertreter der Familie Corethrellidae zum Beispiel holen sich ihre Blutmahlzeit ausschließlich von Fröschen. Während die Morphologie des Nahrungsapparates von für die menschliche Gesundheit relevanten Arten wie Mosquitos gut untersucht ist, haben die „Frosch-stechenden Mücken“ weniger Aufmerksamkeit von der wissenschaftlichen Gemeinschaft erfahren. Besonders die Morphologie des blutsaugenden Rüssels der Corethrellidae wurde nicht mit den neuesten bildgebenden Techniken untersucht. Um dies zu ergänzen, wurden im Zuge dieser Arbeit Dünnschnitte von drei Arten der Gattung *Corethrella* angefertigt und verglichen. Ein 3D-Modell des Rüssels von *C. peruviana* wurde mit Hilfe der Software AMIRA erstellt. Die morphologische Analyse wurde durch Rasterelektronenmikroskopie und Lichtmikroskopie ergänzt. Die Ergebnisse wurden mit Vertretern der anderen blutsaugenden Familien der Zweiflügler Culicidae, Simuliidae, Ceratopogonidae und Psychodidae verglichen, um Ähnlichkeiten und Unterschiede der Morphologie und Funktion des Rüssels zu finden. Die drei untersuchten *Corethrella*-Arten zeigen eine sehr ähnliche Rüssel-Morphologie. Im Vergleich zu den anderen erwähnten Familien sind die Zusammensetzung und Positionierung der Mundwerkzeuge, die den Rüssel aufbauen, auch ähnlich. Eine Ausnahme stellen die Culicidae dar, die Unterschiede im Speichel- und Nahrungskanal aufweisen. Sensillen auf den Labellen und den Maxillarpalpen der drei untersuchten *Corethrella*-Arten werden beschrieben und ihre vermutliche Funktion mit den Resultaten vorbestehender Literatur bei ähnlichen Sensillen anderer Nematocera verglichen. In dieser Arbeit wurde auch die Miniaturisierung der Mundwerkzeuge diskutiert und funktionelle Rahmenbedingungen untersucht, die durch die strukturelle Stabilität der winzigen Stechborsten und die Größe der Blutzellen der Froschwirte entstehen.

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